

The gene cluster involved in aclacinomycin biosynthesis, and its use for genetic engineering

**Field of the invention**

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This invention relates to the gene cluster for aclacinomycin biosynthesis derived from *Streptomyces galilaeus*, and the use of the genes included therein to obtain hybrid antibiotics, or to increase yields of aclacinomycins or related antibiotics.

10 **Background of the invention**

Anthracyclines are widely used anticancer agents. Seven different anthracyclines are in worldwide clinical use: daunorubicin, doxorubicin, idarubicin, epirubicin, pirarubicin, zorubicin and aclarubicin. A representative compound is doxorubicin, being the most efficient and acting on a wide array of malignancies. A variety of toxic effects, like cumulative cardiotoxicity found with doxorubicin has sometimes led to discontinuation of the treatment. Furthermore, there are some type of malignancies which do not respond to available anthracyclines. The mechanism of action of anthracyclines, reflecting to their clinical efficiencies, is not clear, although most researchers consider inhibition of topoisomerase II as a desired effect. Generation of free radicals derived from quinonic structures is suggested to be related to side effects such as cardiotoxicity. Anthracyclines have recently been reviewed by Professor Strohl and his group (1997).

Aclacinomycin A (aclarubicin) first described by Oki *et al.* (1975) is an anthracycline antibiotic produced by *Streptomyces galilaeus* ATCC 31133 and *S. galilaeus* ATCC 31615. It is active against tumor cells and exhibits alleviated toxic properties as compared with doxorubicin. However, its activity does not reach solid tumors, limiting its use in leukemia treatment. Aclarubicin differs from the other counterparts in its structure. A trisaccharide moiety, rhodosamine-2-deoxyfucose-cinerulose A is attached at C-7 by a glycosidic bond, whereas at the corresponding position of daunomycins only one sugar residue, daunosamine, is attached.

Despite the long history of anthracyclines, three decades or so, the studies on their biosynthesis are still going on, and there is further interest to obtain novel molecules for the development of cancer chemotherapeutics. A method currently used for finding novel molecules for drug screening is genetic engineering. Cloning the genes for anthracycline biosynthesis facilitates the production of hybrid anthracyclines, as well as their use in combinatorial biosynthesis to generate novel molecules. As regards the chemical nature of anthracyclines currently in clinical use, aclarubicin has unique features which make its biosynthetic genes interesting in creating novel products.

Regarding the genes for deoxyhexose pathway, Madduri *et al.* (1998) have reported that a gene derived from avermectin biosynthesis cluster caused the production of hybrid anthracyclines altering a sugar moiety when transferred into a *S. peucetius* strain. The product obtained was epirubicin, a commercially important anthracycline. In this case a hydroxy group in the daunosamine moiety was in the opposite stereochemistry due to the action of an avermectin biosynthesis gene.

*S. galilaeus* has been used as the host to prepare hybrid anthracyclines using the genes derived from rhodomycin pathway from *S. purpurascens* (Niemi *et al.*, 1994) and from nogalamycin biosynthesis cluster from *S. nogalater* (Ylihonko *et al.*, 1996a). The genes for nogalamycin pathway were used to generate the hybrid anthracycline production in *S. steffisburgensis* producing typically steffimycin (Kunnari *et al.*, 1997). Previously, biosynthesis genes for actinorhodin have been expressed in *S. galilaeus*, resulting in the formation of aloesaponarin (Strohl *et al.*, 1991). These hybrid compounds were modified in the aglycone moiety. Recently, the biosynthesis genes involved in deoxyhexose pathway of nogalamycin were used to generate hybrid compounds using the *S. galilaeus* mutants as hosts (FI pat. appln No. 982295).

As shown above, *S. galilaeus* has been used as a cloning host to generate novel molecules, whereas its use to donate the genes has not been described. The identified genes involved in aclacinomycin biosynthesis include polyketide reductase gene (Tsukamoto *et al.*, 1994), aklanonic acid methyl ester cyclase (GeneBank, ACCESSION AF043550) and genes for polyketide synthase (Hutchinson and Fujii, 1995; the sequence not available).

## Summary of the invention

The present invention concerns a gene cluster, most of the genes of which are derived from deoxyhexose pathway for rhodosamine, 2-deoxyfucose and/or rhodinosose. The gene cluster  
 5 was cloned from *S. galilaeus* ATCC 31615 and it is involved in biosynthesis of aclacino-  
 mycins.

## Detailed description of the invention

10 The experimental procedures of the present invention include biochemical and chemical  
 methods conventional in the art. Detailed description of the techniques not explained here  
 are given in the manuals by Hopwood *et al.* 'Genetic manipulation of Streptomyces: a  
 laboratory manual'. The John Innes Foundation, Norwich (1985) and by Sambrook *et al.*  
 (1989) 'Molecular cloning: a laboratory manual'.

15 The publications, patents and patent applications cited herein are given in the reference list  
 in their entirety.

The present invention concerns particularly the discovery of the gene cluster for aclacino-  
 20 mycin biosynthesis. The cluster, when introduced into *S. peucetius* strains caused the  
 production of hybrid antibiotics modified in their sugar moiety.

Several strategies may be adopted to clone genes for an antibiotic. Using *E. coli* as a host  
 for a gene library, hybridization is the most advantageous screening strategy. The probe for  
 25 hybridization may be any known fragment that shows sufficient homology to the bio-  
 synthetic cluster for aclarubicin sugars, to be able to hybridize with said cluster. A DNA  
 fragment which is identical to the desired region is preferred. Such a fragment, called Sg-  
 dht, was obtained by PCR amplification of *S. galilaeus* chromosomal DNA, using de-  
 generated oligonucleotides annealing to the conserved region of 4,6-dehydratase gene. 4,6-  
 30 dehydratase is the first enzyme participating to a reaction series that converts a glucose  
 molecule bound to a nucleotide into 6-deoxy sugars generally found in antibiotics. Using  
 this probe it was possible to clone the cluster of deoxyhexose pathway from a restricted

gene library. To simplify the cloning strategy the library was prepared in a pUC-based plasmid (e.g. pBluescript or pWHM1109) replicating in *E. coli*.

The strategy to clone the genes involved in aclacinomycin biosynthesis according to the invention was in brief: Total DNA was isolated from *S. galilaeus* (ATCC 31615) and digested with several restriction enzymes that yield fragments of 10 kb in average. Restriction fragments were analyzed by Southern hybridization using a homologous DNA fragment, Sg-dht, as a probe. *Bgl*II gave a hybridized fragment of 8.5 kb, and a double digestion with *Xho*I and *Not*I gave a hybridized fragment of 7 kb. DNA digestion using (i) *Bgl*II and (ii) *Xho*I-*Not*I was carried out and the fragments were ligated to the *E. coli*-*Streptomyces* shuttle vector, pWHM1109, digested with *Bam*HI and to the pBluescript digested with *Xho*I-*Not*I, respectively. The ligation mixtures were introduced into *E. coli* XL1BlueMRF' that exhibits alleviated restriction-modification systems. Colonies were plated on the agar plates in the dilution to give 200 to 600 cfu (colony forming units) per plate. Well grown colonies were transferred in nylon membranes for hybridization, which was carried out using the Sg-dht probe. Six out of the 786 *Bgl*II-digested clones gave hybridization signal and 7 out of 1523 of those clones carrying *Xho*I-*Not*I fragments. Hybridization and washes were carried out in the stringent conditions of 65°C in a low salt concentration. Several techniques for the labeling of the probe and for hybridization are possible, but the procedure according to Boehringer Mannheim's "The DIG System User's Guide for Filter Hybridization" is preferred. The colonies giving hybridization signals were cultivated for plasmid isolation. The plasmids were analyzed by Southern hybridization to confirm the reliability of the colony hybridization. Plasmids containing the desired DNA fragments (Sg4 and Sg5) were designated as pSgc4 (*Bgl*II-fragment) and pSgc5 (*Xho*I-*Not*I fragment)(see Fig. 2).

The fragments, Sg4 and Sg5, were subcloned for sequencing in *E. coli* vectors pUC19 and pBluescript. In total 30 subclones were used to obtain the nucleotide sequence of Sg4 and Sg5. The sequenced cluster revealed thirteen genes involved in biosynthesis of aclacinomycins. Comparison with the sequences found in the sequence library suggested the functions as *sga*2 for an activator, *sga*3 for a dehydratase, *sga*4 for oxidoreductase, *sga*5 for dTDP-glucose 4,6-dehydratase, *sga*6 for glycosyl transferase (GTF), *sga*7 for a putative

isomerase, *sga8* for aklaviketone reductase, *sga9* for a putative polyketide assembler, *sga10* for a putative cyclase, *sga11* for aminomethylase, *sga12* for glucose-1-phosphate thymidyl transferase, *sga13* for aminotransferase. The function of *sga1* is not suggested based on similarity searches. Based on the deduced functions, nine genes are involved in glycosylation pathway. The genes involved in the formation of aglycone are *sga8*, *sga9*, and *sga10*. The activator, Sga2, may control both the glycosylation system and the formation of aklavinone via polyketide pathway.

Sg4 derived from pSgc4 was cloned in the *Streptomyces* expression vector pIJE486 (Ylihonko *et al.*, 1996b) in *S. lividans* TK24 to give pSgs4. This vector is a high copy number plasmid that replicates in several *Streptomyces* spp. (Ward *et al.*, 1986) and it contains a constitutively expressed promoter, *ermE* (Bibb *et al.*, 1985) upstream from the multiple cloning site. The plasmid pSgs4 isolated from TK24 was introduced into the *S. galilaeus* strains that are blocked in deoxyhexose pathway of aclacinomycin biosynthesis and into the *S. peucetius* mutants producing  $\epsilon$ -rhodomycinone based on a lesion in glycosylation genes. The ability of aclacinomycin production was restored by three *S. galilaeus* mutants, H063, H054 and H065. The mutant strain H063 accumulates aklavinone and it was completely complemented by the plasmid pSgs4. Instead, H054 and H065 producing aklavinone glycosides sharing neutral sugars, but not rhodosamine, were only partially complemented by pSgs4. Surprisingly, H063 carrying pSgs4 (H063/pSgs4) was able to produce aclacinomycins two-fold to that of the wild type *S. galilaeus*. *S. peucetius* M18 and M90 which produce  $\epsilon$ -rhodomycinone were selected to hosts for pSgs4. L-rhamnosyl- $\epsilon$ -rhodomycinone (El Khamed *et al.*, 1977) was obtained when pSgs4 was expressed in the mutants M18 and M90 and, in addition, M18/pSgs4 produced L-daunosaminy- $\epsilon$ -rhodomycinone (Essery and Doyle, 1980). The structures were not new ones but this demonstrates the ability of the gene cluster according to the present invention to generate hybrid products in a heterologous host. To produce hybrid compounds we prefer to use E1 medium supplemented with a suitable antibiotic, in this case, thiostrepton, to maintain the selection pressure for the plasmid containing strains. The products were extracted by organic solvents and purified by chromatography to obtain the compounds in high purity for structural elucidation.

Examples to further illustrate the invention are given hereafter.

### Brief description of the drawings

5 **FIG. 1** shows the structures of aclacinomycin, daunomycin and  $\epsilon$ -rhodomycinone.

**FIG. 2** is a diagram of the gene cluster for aclacinomycin biosynthesis.

**FIG. 3** describes the proposed biosynthesis pathway for sugars found in aclacinomycins.

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**FIG. 4** shows the structures of the hybrid compounds produced by M18/pSgs4 (**1** and **2**) and M90/pSgs4 (**2**).

### EXPERIMENTAL

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#### Materials used

Restriction enzymes used were purchased from Promega (Madison, Wisconsin, USA), Fermentas (Lithuania) or Boehringer Mannheim (Germany), alkaline phosphatase from  
20 Boehringer Mannheim, and used according to manufacturers' instructions. Proteinase K was purchased from Promega and lysozyme from Sigma. Hybond<sup>TM</sup>-N nylon membranes used in hybridization were purchased from Amersham (Buckinghamshire, England), DIG DNA Labelling Kit and DIG Luminescent Detection Kit from Boehringer Mannheim. Qiaquick Gel Extraction Kit from Qiagen (Hilden, Germany) was used for isolating DNA from  
25 agarose.

#### Bacterial strains and their use

*Escherichia coli* XL1BlueMRF' (Stratagene, La Jolla, California) was used for cloning.  
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*Streptomyces lividans* TK24 was the first cloning host for gene expression. The strain was provided by prof. Sir David Hopwood, John Innes Centre, UK.

The wild type, *Streptomyces galilaeus* ATCC 31615, produces aclacinomycins. It was used here to donate the genes of the invention.

*Streptomyces galilaeus* H039 (Ylihonko *et al.*, 1994) produces Akv-(Rho)<sub>0-3</sub>. It was used as an expression host for pSgs4 being more easily transformed than the other mutants or the wild type.

*Streptomyces galilaeus* H054 (Ylihonko *et al.*, 1994) produces Akv-Rho-dF-(CinA)<sub>0-1</sub>, Akv-dF-dF-(CinA)<sub>0-1</sub> and Akv-dF-Rho-Rho. It was used as an expression host for pSgs4.

*Streptomyces galilaeus* H063 produces aklavinone. It is a mutant strain derived from the wild type *S. galilaeus*. H063 was used as an expression host for pSgs4.

*Streptomyces galilaeus* H065 produces aklavinone with neutral glycosides. It is a mutant strain derived from the wild type *S. galilaeus*. H065 was used as an expression host for pSgs4.

*Streptomyces peucetius* M18 and M90 producing  $\epsilon$ -rhodomycinone are the mutants derived from *S. peucetius* var. *caesius* (ATCC 27952). They were used as expression hosts for pSgs4.

## Plasmids

*E. coli* cloning vectors pBluescript SK (Stratagene) and pUC19 (Pharmacia, Sweden) were used for making the subclones for sequencing and pBluescript was used also as a vector of a gene library.

pWHM1109 (provided by prof CR Hutchinson, Wisconsin, USA) is a shuttle vector replicating in *E. coli* and in streptomycetes. It was used as a vector of a gene library.

pIJ486 is a high copy plasmid vector provided by prof. Sir David Hopwood, John Innes Centre, UK (Ward *et al.*, 1986).

pIJE486 (Ylihonko *et al.*, 1996b) is an expression vector containing *ermE* (Bibb *et al.*, 1985) to promote expression of the cloned genes.

### Nutrient media and solutions

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For cultivation of *S. galilaeus* for total DNA isolation TSB medium was used. Lysozyme solution (0.3 M sucrose, 25 mM Tris, pH 8 and 25mM EDTA, pH 8) was used to isolate total DNA. TE buffer (10 mM Tris, pH 8.0 and 1mM EDTA) was used to dissolve DNA.

### 10 TRYPTONE-SOYA BROTH (TSB)

Per litre: Oxoid Tryptone Soya Broth powder 30 g.

### ISP4

Bacto ISP-medium 4, Difco; 37 g/l.

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**E1** Per litre in tap water:

	glucose	20 g
	soluble starch	20 g
	Farmamedia	5 g
20	Yeast extract	2.5 g
	K <sub>2</sub> HPO <sub>4</sub> •3H <sub>2</sub> O	1.3 g
	MgSO <sub>4</sub> •7H <sub>2</sub> O	1 g
	NaCl	3 g
	CaCO <sub>3</sub>	3 g

25 pH adjusted to 7.4 before autoclaving

### General methods:

NMR data was collected with a JEOL JNM-GX 400 spectrometer. <sup>1</sup>H and <sup>13</sup>C NMR  
30 samples were internally referenced to TMS.

The anthracycline metabolites were determined by (i) HPLC (LaChrom, Merck Hitachi, pump L-7100, detector L-7400 and integrator D-7500) using a LiChroCART RP-18 column. Acetonitrile:potassium hydrogen phosphate buffer (60 mM, pH 3.0 adjusted with  
35 citric acid) was used as a mobile phase. Gradient system starting from 65 % to 30 % of



potassium dihydrogen phosphate buffer was used to separate the compounds. The flow rate was 1 ml/min and the detection was carried out at 480 nm, and (ii) by TLC using precoated Kieselgel 60 F<sub>254</sub> glass plates (Merck, Darmstadt, Germany) with an elution solution of toluene:ethyl acetate:methanol:formic acid (50:50:15:3).

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ISP4 plates supplemented with thiostrepton (50 µg/ml) were used to maintain the plasmid carrying cultures.

### Example 1. Cloning the gene cluster for aclacinomycin biosynthesis

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#### 1.1 Selection of clones by hybridization

For isolation of total DNA, *Streptomyces galilaeus* was grown for four days in 50 ml of TSB medium supplemented with 0.5% glycine. The cells were harvested by centrifuging for 15 min (3900 x g) in 12 ml Falcon tubes, and stored at -20°C. Cells from a 50 ml culture were used to isolate DNA. 5 ml of lysozyme solution containing 5 mg/ml of lysozyme was added on the cells of each Falcon tube, and incubated for 20 min at 37°C. 500 µl of 10% SDS containing 0.7 mg of proteinase K was added on the cells, and incubated for 80 min at 62°C, another 500 µl of 10% SDS containing 0.7 mg of proteinase K was added, and incubation was continued for 60 min. The sample was chilled on ice and 600 µl of 3M NaAc, pH 5.8 was added, and the mixture was extracted with equilibrated phenol (Sigma). The phases were separated by centrifuging (1400 x g) for 10 min. The DNA was precipitated from the water phase with an equal volume of isopropanol and collected by spooling with a glass rod and washed by dipping into 70% ethanol, air dried and dissolved in 500 µl of TE-buffer.

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Southern hybridization to determine suitable restriction enzymes for preparing the restricted plasmid libraries was carried out using *Bgl*II, *Xho*I, *Not*I and their combinations. A fragment of about 9 kb hybridizing with the Sg-dht probe was preferred. For hybridization 600 ng of digested *S. galilaeus* DNA was loaded onto the agarose gel and after electrophoresis, the DNA was transferred from the gel to a nylon membrane by vacuum blotting. Hybridization was carried out according to Boehringer Mannheim's manual 'The DIG System User's Guide for Filter Hybridization'. The probe for hybridization, Sg-dht, which was used for

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colony hybridization as well, was obtained by amplifying a gene fragment from the *S. galilaeus* DNA which is internal to the 4,6-dehydratase gene and corresponds to the fragment of 6345 to 6861 shown in SEQ ID NO:14. PCR was used for amplification, and the sequences for the degenerated oligonucleotide primers were 5'-CSGGSGSSGCS-  
 5 GGSTTCATSGG-3' (forward, SEQ. ID. NO:15) and 5'-GGGWRCTGGYRSGGSCCG-TAGTTG-3' (reverse, SEQ. ID. NO:16). Suitable fragments were a 9 kb *Bgl*II fragment and a 7 kb *Xho*I-*Not*I fragment.

Ten micrograms of the chromosomal DNA was digested with *Bgl*II. The DNA fragments  
 10 were separated by agarose gel electrophoresis and the band of 8 to 9 kb were cut from the 0.6% low gelling temperature SeaPlaque® agarose. The DNA band was isolated from the gel using Qiagen Gel Extraction Kit. The isolated fragment was ligated to pWHM1109 plasmid vector digested with *Bam*HI and dephosphorylated, in the ratio of 3 moles of the insert DNA to 1 mole of the vector DNA. The ligated DNA was introduced into *E. coli*  
 15 XL1BlueMRF' by electroporation. Using the whole ligation mixture 786 colonies were obtained. The colonies were grown on agar plates for at least 12 h and transferred to nylon membranes. Hybridization of colony membranes was carried out as Southern using Sg-dht as a probe. Six clones gave signal in hybridization and the corresponding colonies were plated on agar and inoculated in 3 ml of LB medium for isolation of the plasmid DNA.  
 20 Southern hybridization was used to study whether the plasmids derived from the clones carried the desired insert. Four of these plasmids contained the 4,6-dehydratase gene fragment and gave the identical restriction map thus carrying the same fragment representing both orientations. The fragment was designated as Sg4 and the plasmid containing the fragment as pSgc4.

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In the same manner the plasmid library representing a 7 kb *Xho*I-*Not*I DNA fragment derived from *S. galilaeus* was constructed. pBluescript was digested with *Xho*I-*Not*I and the library containing the gene fragments of around 7 kb was constructed. In total 1523 colonies were hybridized and seven turned to be the desired clone. As described above, the  
 30 clones were studied for the *Xho*I-*Not*I fragment. The insert fragment was designated as Sg5 and the plasmid as pSgc5. The strain *E. coli* XL1Blue MRF'/pSgc5 obtained was deposited according to the rules of the Budapest Treaty at Deutsche Sammlung von Mikroorganismen

und Zellkulturen GmbH (DSMZ) on August 12, 1999 with the accession number DSM 12999. The fragments Sg4 and Sg5 overlap within 836 bp corresponding bases from 6181 to 7016 in SEQ ID NO:14.

## 5 1.2. Subcloning the fragments for sequencing

To determine the nucleotide sequence of the whole cluster of the Sg4 and Sg5 suitable subclones were constructed. The convenient restriction sites were used for subcloning the 14806 bp region in the plasmids pUC19 and pBluescript. Nineteen subclones were needed to sequence Sg4, and 11 subclones for Sg5.

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*E. coli* XL1BlueMRF' cells containing the subcloned plasmids were cultivated overnight at 37°C in 5 ml of LB-medium supplemented with 50 µg/ml of ampicillin. To isolate plasmids for sequencing reactions Wizard Plus Minipreps DNA Purification System kit of Promega or Biometra Silica Spin Disc Plasmid DNA Miniprep kit of Biomedizinische Analytik GmbH were used according to the manufacturers' instructions.

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DNA sequencing was performed using the automatic ABI DNA sequencer (Perkin-Elmer) according to the manufacturer's instructions.

## 20 1.3 Sequence analysis and the deduced functions of the genes

Sequence analyses were made using the GCG sequence analysis software package (Version 8; Genetics Computer Group, Madison, Wis., USA). The translation table was modified to accept also GTG as a start codon. Codon usage was analyzed using published data (Wright and Bibb 1992).

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According to the CODONPREFERENCE program the sequenced DNA fragment revealed 11 complete open reading frames (ORFs), and two 5' ends of the other ORFs (*sga1* and *sga13*). The functions of the genes were concluded by comparing the amino acid sequences translated from their base sequences to the known sequences in the data banks. The results are shown in Table 1 referring to the sequence data given in the application.

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The suggested functions for the genes match well with a proposed biosynthetic pathway of sugars of aclacinomycins (Fig. 3). The last residue in a trisaccharide moiety of aclacinomycins is rhodnose that is enzymatically converted to cinerulose. Aclacinomycin N, a precursor of aclarubicin, contains rhodnose as the third sugar residue.

**Table 1.**

Gene	Position	Amino acids	Deduced function	Remarks
<i>sga1</i>	-1986 compl	>662	unknown	not complete Seq.ID.NO:1
<i>sga2</i>	2523-3341	272	activator	Seq.ID.NO:2
<i>sga3</i>	3355-4659 compl	434	dehydratase	Seq.ID.NO:3
<i>sga4</i>	4821-5810	329	oxidoreductase	Seq.ID.NO:4
<i>sga5</i>	5920-6891 compl	323	dTDP-glucose 4,6-de- hydratase	Seq.ID.NO:5
<i>sga6</i>	6879-8210 compl	443	glycosyl transferase (GTF)	Seq.ID.NO:6
<i>sga7</i>	8287-9618 compl	443	putative isomerase	Seq.ID.NO:7
<i>sga8</i>	9642-10445 compl	267	aklaviketone reductase (KR11)	Seq.ID.NO:8
<i>sga9</i>	10471- 10905 compl	144	putative polyketide assembler	Seq.ID.NO:9
<i>sga10</i>	11115- 11894	259	putative cyclase	Seq.ID.NO:10
<i>sga11</i>	11956- 12672	238	aminomethylase	Seq.ID.NO:11
<i>sga12</i>	12685- 13560 compl	291	glucose-1-phosphate thymidyltransferase	Seq.ID.NO:12
<i>sga13</i>	13783- 14805	341	aminotransferase	Seq.ID.NO:13 not complete

#### 1.4 Expression cloning in *Streptomyces* strains

The 8 kb *Bam*HI-*Hind*III fragment from pSgc4 was ligated in pIJE486 to give pSgs4. Plasmid pSgs4 was introduced into *S. lividans* TK24 by protoplast transformation. The strain *S. lividans* TK24/pSgs4 obtained was deposited according to the rules of the  
 5 Budapest Treaty at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) on August 12, 1999 with the accession number DSM 12998. The plasmid pSgs4 was isolated from the strain, and further transferred into *S. galilaeus* mutant H039. The plasmid prepareate isolated from H039 was subsequently introduced into H063, H054, and H065 mutants deficient of glycosylation system of aclacinomycins. The usage of H039 as a  
 10 primary *S. galilaeus* host was due to the better efficiency for the intake of foreign DNA.

*S. galilaeus* mutants were studied for complementation by cultivating the clones containing pSgs4 in E1 medium supplemented with thiostrepton (10  $\mu$ g/ml). The products from a 500  $\mu$ l sample of the culture broth were extracted with toluene:methanol (1:1) at pH 7. The  
 15 metabolites from both the transformed clones and the mutants were analyzed by TLC and HPLC to find the differencies caused by pSgs4. H063 producing endogenously aklavinone was restored to aclacinomycin producer with pSgs4. No aklavinone was found in the culture broth of H063/pSgs4. However, complementation was not completed when pSgs4 was expressed in H054 and H065. Both of the mutants produce aklavinone with neutral  
 20 glycosides. Incomplete complementation was presumably due to the loss of the plasmids of some bacterial cells during cultivation, or a low expression of the genes needed as an activator is not present in pSgs4.

In the same manner, pSgs4 isolated from TK24 was introduced into the *S. peucetius*  
 25 mutants M18 and M90. The characteristic product for these mutants is  $\epsilon$ -rhodomycinone. The strains M18/pSgs4 and M90/pSgs4 containing the plasmid were cultivated in E1 medium supplemented with thiostrepton (10  $\mu$ g/ml), and the metabolites therein were analyzed by TLC and HPLC. Both of the clones revealed an altered production profile as compared with the products obtained from the mutants. M90/pSgs4 accumulated a  
 30 glycosylated product, yielding  $\epsilon$ -rhodomycinone as the aglycone. The compound was identified as L-rhamnosyl- $\epsilon$ -rhodomycinone which has been previously synthesized (CAS=63252-11-9) by El Khamed *et al.* (1977).

M18/pSgs4 produced two compounds differing from the parental strain. According to the HPLC and TLC data one compound was the same as was produced by M90/pSgs4, L-rhamnosyl- $\epsilon$ -rhodomycinone, and the other one was L-daunosaminyl- $\epsilon$ -rhodomycinone, which was previously characterized by Essery and Doyle (1980).

**Table 2:** TLC and HPLC data of the hybrid products

Product	Rf-value	Retention time
$\epsilon$ -rhodomycinone	0.67	6.70
L-rhamnosyl- $\epsilon$ -rhodomycinone	0.38	5.00
L-daunosaminyl- $\epsilon$ -rhodomycinone	0.04	4.06

### 1.5 Applicability of pSgs4 for strain improvement

Since H063 was completely complemented by pSgs4, the production level of aminoglycosides was studied. For this purpose, H063/pSgs4, H063 and the wild type *S. galilaeus* were cultivated in E1 medium in the Erlenmeyer bottles for four days. Two samples of 2 ml from each culture were extracted first with toluene:methanol (1:1) in acidic conditions to remove the neutral glycosides and the aglycones. The extraction procedure was repeated until neutral glycosides and the aglycones had disappeared from the water phase. The amount of anthracycline metabolites in toluene phase was determined and is shown in Table 3. Aclacinomycins containing rhodosamine were extracted from the water phase by chloroform. Both toluene and chloroform extracts were analyzed by TLC and toluene phases contained mostly aklavinone and the degradative products. Chloroform phases contained mainly aminoglycosides, although minor amounts of the aglycones were also detected. Extracts were evaporated to dryness and subsequently dissolved into 1 ml of methanol. The amounts of anthracycline metabolites were detected by spectrophotometer at 430 nm. The amounts related to absorbance were calculated using an extinction coefficient of 13000. The results given as mg/l of cultivation broth are shown in Table 3. The production of aclacinomycins by H063/pSgs4 was at least twofold better than obtained by the wild type.

**Table 3.**

Sample	Chloroform phase aminoglycoside fraction		Toluene phase aglycone fraction	
	Absorbance	Concentration (mg/l)	Absorbance	Concentration (mg/l)
H063	0.401	12.6	2.956	92.3
H063/pSgs4	2.751	85.9	2.974	92.9
<i>S. galilaeus</i>	1.338	41.8	0.690	21.5

- 10 The ability to increase the yield of aclacinomycins by pSgs4 in the mutant H063 suggests that the genes according to the present invention are useful in strain improvement.

#### **Example 2. Compounds generated by pSgs4**

- 15 The seed culture, 180 ml of E1 culture of the plasmid containing strains, M18/pSgs4 or M90/pSgs4, was obtained by cultivating each of the strains in three 250 ml Erlenmeyer flasks containing 50 ml of E1-medium supplemented with thiostrepton (5 µg/ml) for four days at 30°C, 330 rpm. The combined culture broths (180 ml) were used to inoculate 13 l of E1-medium in a fermentor (Biostat E). Fermentation was carried out for five days at  
20 28°C (330 rpm, aeration: 450 l/min).

The cells were harvested by centrifuging. 2.6 l of methanol was used to brake the bacterial cells. The anthracycline metabolites were extracted from methanol solution at pH 8 using 2 l of ethyl acetate and the extract was evaporated to dryness. The viscous residue was loaded  
25 onto a silica column of 4 × 10 cm and toluene:ethyl acetate:formic acid (50:50:3) with increasing amount of methanol was used as an eluent. Pure fractions were pooled and extracted with 1M phosphate buffer (pH 8.0) and water. Organic phase was dried with anhydrous Na<sub>2</sub>SO<sub>4</sub> and then treated with hexane to effect precipitation. Pure compounds appeared as red powders dried under vacuum.

Complete structural determination of the compounds were accomplished by NMR. Proton and carbon assignments were based on a conventional NOE difference, pHSQC and HMBC measurements. Connectivities in particular relied heavily on HMBC experiment.

- 5 As deduced from the data given in Table 4, the structures revealed were L-rhamnosyl- $\epsilon$ -rhodomycinone (**1**) and L-daunosaminy- $\epsilon$ -rhodomycinone (**2**) shown in Figure 4.

- Although these structures were not novel, the generation of the hybrid products by the genes involved in glycosylation portion of aclacinomycin biosynthesis well demonstrates  
10 that the genes of pSgs4 are functional and ready to use in drug discovery for finding novel molecules.

### Deposited microorganisms

- 15 The following microorganisms were deposited according to the Budapest Treaty at Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Mascheroder Weg 1b, D-38124 Braunschweig, Germany.

Microorganism	Accession number	Date of deposit
20 <i>S. lividans</i> TK24/pSgs4	DSM 12998	12 August 1999
<i>E. coli</i> XL1BlueMRF'/pSgc5	DSM 12999	12 August 1999



**Table 4.**  $^1\text{H}$  and  $^{13}\text{C}$  chemical shifts of **1** ( $\text{DMSO}_{\text{d}_6}$ ) and **2** (trace of TFA in  $\text{DMSO}_{\text{d}_6}$ ) in 400 and 100 MHz, respectively.

Site	<b>1</b>		<b>2</b>	
	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$
1	7.74, 1H, dd, 7.5, 0.9	118.9(d)	7.74, 1H, dd, 7.5, 1.0	119.7(d)
2	7.64, 1H, dd, 8.4, 7.5	136.5(d)	7.68, 1H, dd, 8.1, 7.5	137.4(d)
3	7.22, 1H, dd, 8.4, 0.9	124.1(d)	7.24, 1H, dd, 8.1, 1.0	125.0(d)
4	-	161.8(s)	-	162.6(s)
4-OH	12.00, 1H, s	-	exchange broadened	-
4a	-	115.2(s)	-	115.9(s)
5	-	189.9(s)	-	190.6(s)
5a	-	110.4(s)	-	111.4(s)
6	-	156.2(s)	-	157.1(s)
6-OH	13.41, 1H, s	-	exchange broadened	-
6a	-	135.1(s)	-	135.7(s)
7	5.14, 1H, d, 4.5	70.9(d)	5.15, 1H, d, 3.6	71.3(d)
8A	2.31, 1H, d, 15.1	28.9(t)	2.33, 1H, d, 14.6	34.0(t)
8B	2.14, 1H, dd, 15.1, 4.5	-	2.21, 1H, dd, 14.6, 3.8	-
9	-	70.0(s)	-	70.9(s)
10	4.16, 1H, s	51.2(d)	4.23, 1H, s	51.8(d)
10a	-	134.8(s)	-	136.1(s)
11	-	156.0(s)	-	156.8(s)
11-OH	12.77, 1H, s	-	exchange broadened	-
11a	-	110.8(s)	-	111.1(s)
12	-	185.4(s)	-	186.0(s)
12a	-	132.6(s)	-	133.3(s)
13A	1.73, 1H, dq, 13.9, 7.4	31.7(t)	1.83, 1H, dq, 14.1, 7.3	32.0(t)
13B	1.38, 1H, dq, 13.9, 7.4	-	1.47, 1H, dq, 14.1, 7.3	-
14	1.05, 3H, t, 7.4	6.09(q)	1.13, 3H, t, 7.3	6.90(q)
15	-	170.4(s)	-	171.1(s)
16	3.63, 3H, s	51.7(q)	3.70, 3H, s	52.3(q)
1'	5.28, 1H, brs	103.7(d)	5.52, 1H, d, 3.1	100.7(d)
2'	3.83, 1H, d, 5.2	70.9(d)	2.18, 2H, m	27.1(t)
3'	3.44, 1H, dd, 9.0, 5.2	70.8(d)	3.40, 1H, dd, 11.8, 5.1	55.5(d)
4'	3.41, 1H, dd, 9.1, 9.0	72.0(d)	3.98, 1H, brs	67.0(d)
5'	3.77, 1H, dq, 9.1, 6.2	68.9(d)	4.21, 1H, q, 6.3	65.3(d)
6'	1.29, 3H, d, 6.2	16.9(q)	1.32, 3H, t, 6.3	16.7(q)

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